Integrin α3β1 Potentiates TGFβ-Mediated Induction of MMP-9 in Immortalized Keratinocytes

John M. Lamar[1,2], Vandana Iyer[1,2] and C. Michael DiPersio[1]

Transforming growth factor-β (TGFβ) signaling pathways regulate a number of keratinocyte functions during epidermal carcinogenesis and wound healing, including proliferation, survival, and migration. TGFβ can induce expression of the matrix metalloproteinase-9 (MMP-9), which has critical roles in promoting extracellular matrix remodeling and angiogenesis during tumorigenesis and tissue repair. Integrin α3β1 is a cell adhesion receptor for laminin-332/laminin-5 with important roles in the survival and motility of epidermal keratinocytes. We previously reported that α3β1 induces the expression of MMP-9 in immortalized keratinocytes. In this study, we show that endogenous TGFβ is required for maximal MMP-9 expression, and that α3β1 is required for full induction of MMP-9 protein and mRNA in response to TGFβ. This regulation was not observed in non-immortalized, primary keratinocytes, indicating that coordinate regulation of MMP-9 by α3β1 and TGFβ is a property of immortalized cells. α3β1 did not regulate endogenous TGFβ gene expression, TGFβ bioavailability, or TGFβ-Smad signaling. However, the combined inductive effects of TGFβ and α3β1 on MMP-9 were suppressed by a Src family kinase (SFK) inhibitor, indicating involvement of a SFK pathway. These findings provide early evidence of a role for α3β1 in augmenting TGFβ-mediated induction of MMP-9 in immortalized or transformed keratinocytes during skin carcinogenesis.

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INTRODUCTION

Members of the transforming growth factor-β (TGFβ) family regulate diverse cell functions, including differentiation, proliferation, survival, and migration (ten Dijke and Hill, 2004). TGFβ has important, although paradoxical roles in epidermal carcinogenesis. TGFβ acts as a tumor suppressor at early stages of skin tumorigenesis (Wang, 2001; Zavadil and Bottinger, 2005). However, it promotes keratinocyte epithelial-to-mesenchymal transition (EMT) and malignant conversion of tumor cells at later stages (Kane *et al.*, 1991; Levine *et al.*, 1993; Wang, 2001; Han *et al.*, 2005), a function that is thought to require sustained TGFβ signaling through autocrine stimulation of TGFβ expression (Zavadil and Bottinger, 2005). These paradoxical roles for TGFβ suggest a switch in its function that occurs during tumor progression. Consistently, the repertoire of cellular responses to TGFβ changes dramatically during EMT and tumor progression (Derynck *et al.*, 2001; Wakefield and Roberts, 2002). Expression of TGFβ by epidermal keratinocytes is also increased during cutaneous wound healing (Kane *et al.*, 1991; Levine *et al.*, 1993; Han *et al.*, 2005), where it contributes to the regulation of keratinocyte motility (Singer and Clark, 1999).

Each of the three mammalian TGFβ isoforms, TGFβ1, -2, and -3, is secreted as an inactive complex that contains the latency-associated protein and latent TGFβ binding protein (Sheppard, 2005). This latent complex is linked to the extracellular matrix (ECM) through latent TGFβ binding protein (Taipale *et al.*, 1994), and it can be activated through cleavage by extracellular proteases (Lyons *et al.*, 1990; Sato *et al.*, 1990; Yu and Stamenkovic, 2000), conformational changes induced by interactions with thrombospondin-1 (Schultz-Cherry *et al.*, 1995; Crawford *et al.*, 1998), or binding of integrin αvβ6 to an arginine–glycine–aspartic motif on latency-associated protein (Munger *et al.*, 1999; Annes *et al.*, 2004; Sheppard, 2005). Once activated, TGFβ interacts with type I and type II serine/threonine kinase receptors to initiate signaling pathways that modulate gene expression. While many responses to TGFβ appear to be mediated through the Smad family of transcription factors, Smad-independent mechanisms of TGFβ signaling also exist (Derynck and Zhang, 2003). Indeed, TGFβ signaling through mitogen-activated protein kinases plays important roles in post-transcriptional mRNA stability (Dibrov *et al.*, 2006). Thus, cellular responses to TGFβ depend on a balance of transcriptional and post-transcriptional regulation through both Smads and mitogen-activated protein kinases, and this

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Abbreviations: ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; ERK, extracellular signal-regulated kinase; LAP, latency-associated protein; LN-332, laminin-332/laminin-5; LTBP, latent TGFβ binding protein; MEK, MAPK/ERK kinase; MK, mouse keratinocyte; MLEC, mink lung epithelial cell; MMP-9, matrix metalloproteinase-9; TGFβ, transforming growth factor-β

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regulation is highly context-dependent (Zavadil *et al.*, 2001; Derynck and Zhang, 2003; Moustakas and Heldin, 2005; Zavadil and Bottinger, 2005).

TGFβ signaling is often coupled with alterations in cell adhesion and motility, and TGFβ-induced EMT in keratinocytes is accompanied by changes in the expression of genes that are involved in these processes (Zavadil *et al.*, 2001). One target of TGFβ signaling in both normal and transformed keratinocytes, as well as other carcinoma cells, is the matrix metalloproteinase-9 (MMP-9)/gelatinase B (Salo *et al.*, 1991; Weeks *et al.*, 2001; Santibanez *et al.*, 2002; Kalembeyi *et al.*, 2003; Safina *et al.*, 2007). MMP-9 has critical roles in ECM remodeling and angiogenesis (McCawley and Matrisian, 2001), and its induction by TGFβ is likely to be important during both skin carcinogenesis and cutaneous wound healing.

Integrin receptors for the ECM also play important regulatory roles in epidermal tumorigenesis and wound healing (Grose *et al.*, 2002; Watt, 2002), and recent studies have implicated certain integrins in regulating some TGFβ-mediated effects on cell motility and EMT (Bhowmick *et al.*, 2001; Jeong and Kim, 2004; Kloeker *et al.*, 2004). Integrin α3β1 is a major receptor for keratinocyte adhesion to laminin-332/laminin-5 (LN-332) in the cutaneous basement membrane (Carter *et al.*, 1991; Delwel *et al.*, 1994; Kreidberg, 2000), and it is implicated in wound re-epithelialization through its established roles in regulating basement membrane organization and keratinocyte motility (DiPersio *et al.*, 1997; deHart *et al.*, 2003; Choma *et al.*, 2004; Frank and Carter, 2004). α3β1 is also expressed at high levels in epidermal tumors and other carcinomas where it may regulate tumor cell invasion and metastasis (Natali *et al.*, 1993; Bartolazzi *et al.*, 1994; Melchiori *et al.*, 1995; Patriarca *et al.*, 1998; Lohi *et al.*, 2000; Morini *et al.*, 2000; Felding-Habermann, 2003). We recently reported that α3β1 induces MMP-9 expression in immortalized keratinocytes by promoting mRNA stability (Iyer *et al.*, 2005), a mechanism that bears intriguing similarities to the previously described role for TGFβ in promoting MMP-9 mRNA stability in prostate cancer cells (Sehgal and Thompson, 1999). Interestingly, MMP-9 induction by TGFβ (Sehgal and Thompson, 1999) or α3β1 (DiPersio *et al.*, 2000) was acquired by immortalized or transformed cells, suggesting that cells undergoing EMT may activate new pathways that promote MMP-9 expression in response to integrins and/or growth factors. Although there are several reports that TGFβ regulates motility of keratinocytes or carcinoma cells through alterations in α3β1 expression or function (Giannelli *et al.*, 2002; Decline *et al.*, 2003; Jeong and Kim, 2004), cooperative roles for TGFβ and α3β1 in the regulation of MMP-9 have not been explored.

Previously, we showed that ablating integrin α3β1 expression by null mutation of the α3 *Itga3*, gene leads to greatly reduced levels of MMP-9 mRNA in immortalized keratinocytes, revealing an α3β1-dependent mechanism of MMP-9 induction in these cells (Iyer *et al.*, 2005). In this study, we provide evidence of a role for α3β1 in potentiating the TGFβ-mediated induction of MMP-9. We show that inhibiting endogenous TGFβ with a function blocking antibody abrogates MMP-9 mRNA expression in immortalized keratinocytes that express α3β1. We further demonstrate that α3β1-dependent MMP-9 expression in these cells is not due to α3β1-dependent differences in endogenous TGFβ gene expression, TGFβ bioavailability, or TGFβ-mediated Smad signaling. Instead, our results show that α3β1 potentiates the induction of MMP-9 in response to TGFβ through a mechanism that requires Src family kinase (SFK) signaling. α3β1 and TGFβ also cooperatively enhanced MMP-9 induction in response to oncogenically activated Ras, a known stimulator of MMP-9 gene transcription. These findings may reflect an important role for α3β1 in augmenting specific TGFβ-mediated cellular responses during epidermal tumorigenesis or other epidermal remodeling processes that involve MMP-9.

RESULTS

TGFβ promotes MMP-9 mRNA expression in immortalized keratinocytes

TGFβ has been shown to induce MMP-9 expression in normal human keratinocytes (Salo *et al.*, 1991). Therefore, we first tested the role of TGFβ in the regulation of MMP-9 expression in immortalized mouse keratinocytes (MK cells). MK cells were cultured on laminin-332-rich extracellular matrix (LN-332 ECM) for 24 hours in the presence of increasing concentrations of exogenous TGFβ. Treatment with TGFβ caused a dose-dependent increase in MMP-9 mRNA levels, while β-actin mRNA levels remained constant at all doses tested (Figure 1a). Since epidermal keratinocytes produce TGFβ during wound healing (Kane *et al.*, 1991), we also tested the effect of inhibiting endogenous TGFβ on MMP-9 mRNA expression. Treatment of MK cells for 24 hours with an anti-TGFβ antibody that neutralizes TGFβ function caused a dose-dependent decrease in MMP-9 mRNA levels (Figure 1b). As a control, treatment with an isotype-specific control immunoglobin G at the highest concentration had no effect (Figure 1b). Approximately 60% inhibition of MMP-9 mRNA levels was achieved by treatment with 30 μg/ml anti-TGFβ blocking antibody (Figure 1c). Together, these results indicate that TGFβ promotes MMP-9 mRNA expression in immortalized keratinocytes, and that endogenous TGFβ function is required for full levels of MMP-9 expression.

We showed previously that α3-null MK cells (MK−/− cells), which lack integrin α3β1, show greatly reduced MMP-9 mRNA expression compared with wild-type MK cells (MK+/+ cells), indicating that α3β1 is required for MMP-9 expression (Iyer *et al.*, 2005). To test if exogenously provided TGFβ can induce MMP-9 expression in the absence of α3β1, MK−/− cells were cultured on LN-332 ECM with increasing concentrations of TGFβ. Treatment of MK−/− cells with exogenous TGFβ for 24 hours induced a dose-dependent increase in MMP-9 mRNA, while β-actin mRNA levels were unaffected (Figure 2a). These findings were confirmed by quantitative real-time reverse transcriptase PCR (RT-PCR) (Figure 2b), and they show that exogenous TGFβ can induce MMP-9 mRNA in the absence of integrin α3β1 when it is present at sufficiently high concentrations.

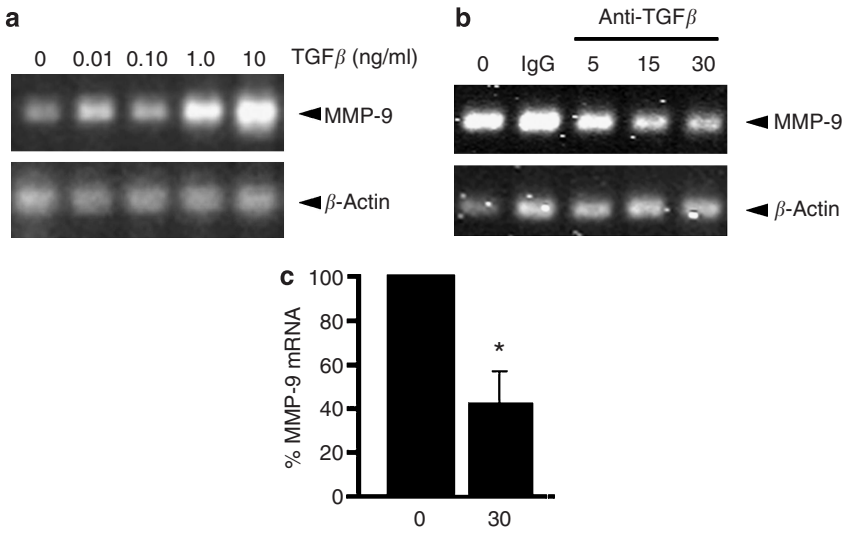

Figure 1. TGFβ promotes MMP-9 mRNA expression in immortalized keratinocytes. Immortalized keratinocytes (MK+/+ cells) were cultured on LN-332 ECM for 24 hours in serum-free medium, then treated with either (**a**) increasing doses of active TGFβ as indicated, or (**b**) increasing concentrations of anti-TGFβ neutralizing antibody (0, 5, 15, or 30 μg/ml) or control immunoglobin G (30 μg/ml) as indicated. For both (**a**) and (**b**), total RNA was isolated and RT-PCR was performed to assess MMP-9 and β-actin mRNA levels. (**c**) Quantification of MMP-9 mRNA levels in MK cells cultured in the absence (0) or presence (30) of 30 μg/ml anti-TGFβ neutralizing antibody. Average MMP-9 mRNA levels, after normalization to β-actin mRNA, are shown as a percentage of levels in untreated cells. Data are the mean ± SEM; $n = 3$; $^*P < 0.05$, *t*-test.

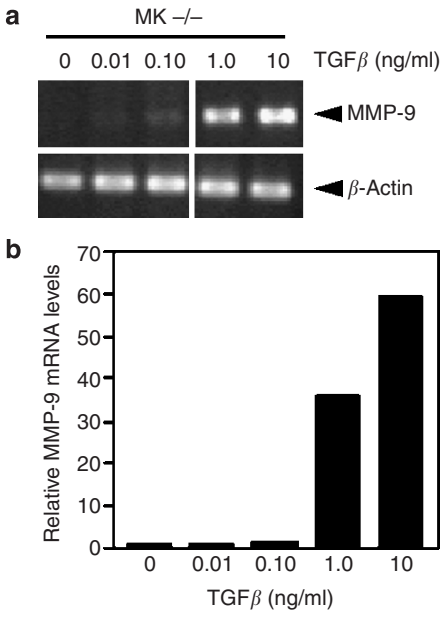

Figure 2. Exogenous TGFβ induces a dose-dependent increase in MMP-9 mRNA expression independently of integrin α3β1. (**a**) α3-null MK−/− cells were cultured on LN-332 ECM for 24 hours in serum-free medium in the presence of increasing doses of exogenous TGFβ, as indicated. RT-PCR was performed to assess MMP-9 and β-actin mRNA levels. (**b**) MK−/− cells were treated as in (**a**), and real-time RT-PCR was performed to quantify relative MMP-9 mRNA levels after normalization to β-actin mRNA.

It is well established that TGFβ can promote post-transcriptional mRNA stability (reviewed in Dibrov *et al.*, 2006), and it has been shown to induce MMP-9 mRNA in prostate carcinoma cells through enhanced mRNA stability without increasing transcription from the MMP-9 promoter (Sehgal and Thompson, 1999). Similarly, it has been shown that TGFβ did not stimulate the MMP-9 promoter in some epidermal cell lines (Santibanez *et al.*, 2002). We used luciferase reporter assays to show that TGFβ failed to induce transcriptional activity of a 1.9 kb human MMP-9 promoter fragment (Shimajiri *et al.*, 1999), either in the α3-null MK−/− cells or in MK−/− cells that were stably transfected with human α3 integrin (MKα3 cells) (Figure 3a, graph). However, TGFβ did induce endogenous MMP-9 expression above basal levels in these same cells, consistent with post-transcriptional induction (Figure 3a, gels). The higher basal level of endogenous MMP-9 in MKα3 cells is due to α3β1-dependent induction of MMP-9 mRNA that we described previously (Iyer *et al.*, 2005). The apparent cooperative effect of TGFβ and α3β1 on MMP-9 protein expression is explored later in this study (see Figure 7). As a positive control for stimulation of the MMP-9 promoter, adenoviral expression of an activated Ras mutant, RasV12, similarly stimulated the MMP-9 promoter in both MK−/− cells and MKα3 cells (Figure 3b, graph). As expected, this promoter induction was not accompanied by increased MMP-9 levels in the MK−/− cells (Figure 3b, gels), since α3β1 is required for post-transcriptional stability and subsequent accumulation of the MMP-9 mRNA transcript (Iyer *et al.*, 2005).

α3β1-dependent MMP-9 expression does not result from changes in endogenous TGFβ gene expression, TGFβ secretion, or bioavailability of ECM-associated TGFβ

α3β1 is required for MMP-9 expression in MK cells (Iyer *et al.*, 2005), and our current results show that blocking endogenous TGFβ reduces MMP-9 mRNA in α3β1-expressing MK cells (Figure 1b and c), while treatment with exogenous TGFβ increases MMP-9 mRNA in α3β1-deficient MK−/− cells

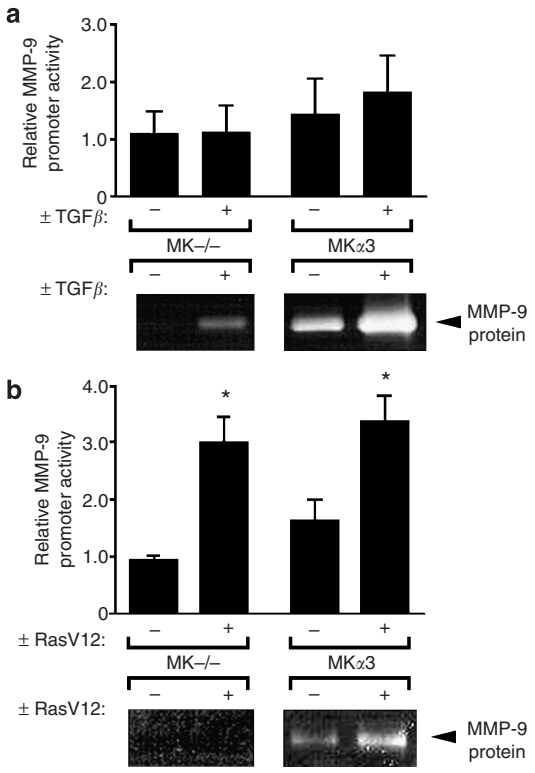

Figure 3. TGFβ does not induce the MMP-9 promoter in MK cells. (**a**) MK−/− cells or MKα3 cells grown on LN-332 ECM were transfected with an MMP-9 promoter/luciferase reporter plasmid and cultured for 48 hours in serum-free medium in the absence or presence of 10 ng/ml TGFβ, as indicated. (**b**) MK−/− cells or MKα3 cells grown on LN-332 ECM were left uninfected (−RasV12) or infected with RasV12 adenovirus (+RasV12), then transfected with the MMP-9 promoter/*luciferase* reporter plasmid and cultured for an additional 48 hours in serum-free medium. For (**a**) and (**b**), cell lysates were assayed for luciferase expression (arbitrary units). Data are the mean ± SEM; $n = 3$–6 in at least two separate experiments; $^*P < 0.01$, one-way analysis of variance with Newman–Keuls *post hoc* test. Culture media from the transfected cells were also collected and assayed for endogenous MMP-9 protein by gelatin zymography (gels).

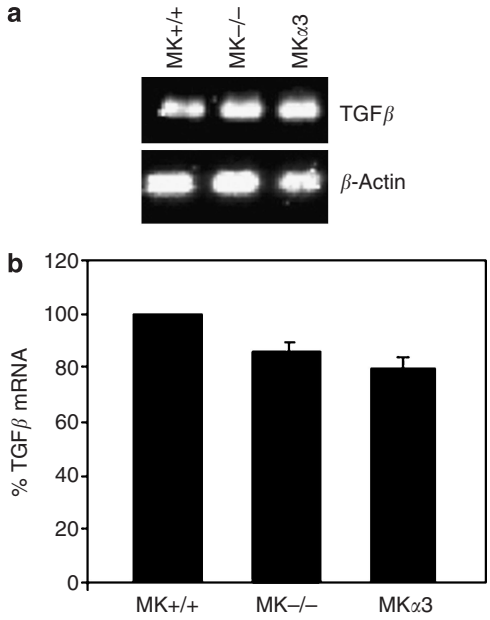

Figure 4. TGFβ gene expression in MK cells is not regulated by integrin α3β1. (**a**) MK+/+, MK−/−, or MKα3 cells were cultured on LN-332 ECM in serum-free medium for 24 hours, then RT-PCR was performed to assess TGFβ and β-actin mRNA levels. (**b**) Quantification of TGFβ mRNA levels, after normalization to β-actin mRNA, is shown as a percentage of levels in MK+/+ cells. Data are mean ± SEM; $n = 5$, from three independent experiments.

(Figure 2). Together, these findings raised the possibility that α3β1 regulates MMP-9 expression by controlling endogenous TGFβ gene expression. To test this possibility, we compared TGFβ mRNA levels in MK+/+ cells, MK−/− cells, or the α3-transfected MKα3 cells. When cultured on LN-332 ECM under conditions where MMP-9 expression is known to be α3β1-dependent (Iyer *et al.*, 2005), MK cells expressed similar amounts of TGFβ mRNA regardless of α3β1 expression (Figure 4a and b), indicating that α3β1-dependent MMP-9 expression is not due to α3β1-mediated differences in TGFβ gene expression.

We next tested whether differential secretion of TGFβ into the culture medium by MK+/+ cells and MK−/− cells could account for α3β1-dependent MMP-9 expression. First, we tested if treatment with serum-free, conditioned culture medium from MK+/+ cells can induce MMP-9 expression in MK−/− cells. These experiments were performed over a 24-hour treatment period, since experiments in which we either blocked endogenous TGFβ (Figure 1) or added exogenous TGFβ (Figure 2) indicated that this time frame would be sufficient to see effects on MMP-9 expression. MK+/+ cell culture medium failed to induce MMP-9 mRNA in MK−/− cells (Figure 5, lane 1), while treatment of the same MK−/− cells with exogenous TGFβ induced MMP-9 mRNA expression (Figure 5, lane 4), indicating that active TGFβ was not present at sufficiently high concentrations in MK+/+ cell culture medium to induce MMP-9 mRNA. This finding was confirmed using ELISA to quantify TGFβ levels in the culture medium (data not shown), which showed that the amount of secreted TGFβ that had accumulated within 24 hours from either α3β1-expressing (i.e. MK+/+ or MKα3) or α3β1-deficient (i.e. MK−/−) cells was at least 20-fold lower than the minimum concentration of exogenous TGFβ (1 ng/ml) that was sufficient to induce MMP-9 mRNA in the MK−/− cells (see Figure 2).

The low level of endogenous TGFβ that was detected in MK cell culture medium is consistent with the fact that most TGFβ is secreted from cells as part of an inactive latent complex that is covalently associated with the ECM through latent TGFβ binding protein (Sheppard, 2005). It therefore seemed possible that α3β1 somehow regulates the bioavailability of ECM-associated TGFβ, as has been shown for integrin αvβ6 (Munger *et al.*, 1999; Annes *et al.*, 2004; Sheppard, 2005). To explore this possibility, we exploited transformed mink lung epithelial cells (MLECs) that stably express a *luciferase* reporter gene under control of the TGFβ-responsive plasminogen activator inhibitor-1 (PAI-1) promoter (Abe *et al.*, 1994). Since MLECs are not capable of activating latent TGFβ, luciferase reporter expression in these cells is a useful readout to assess the level of active TGFβ that is associated with the ECM (Annes *et al.*, 2004).

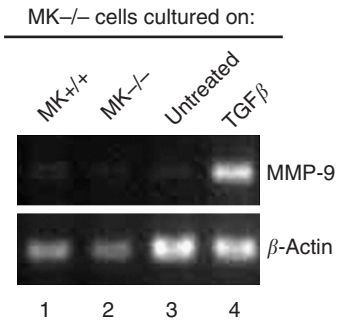

Figure 5. The level of endogenous TGFβ in MK+/+ cell culture medium is not sufficiently high to induce MMP-9 mRNA. MK−/− cells were cultured on LN-332 ECM for 24 hours in serum-free culture medium that was conditioned by MK+/+ cells (lane 1), as described in Materials and Methods. As negative controls, MK−/− cells were similarly treated with their own conditioned medium (lane 2) or left untreated (lane 3). As a positive control, MK−/− cells were treated with 1 ng/ml TGFβ (lane 4). RT-PCR was performed to assess MMP-9 and β-actin mRNA levels. Results shown are representative of three independent experiments and were also confirmed by real-time RT-PCR (data not shown).

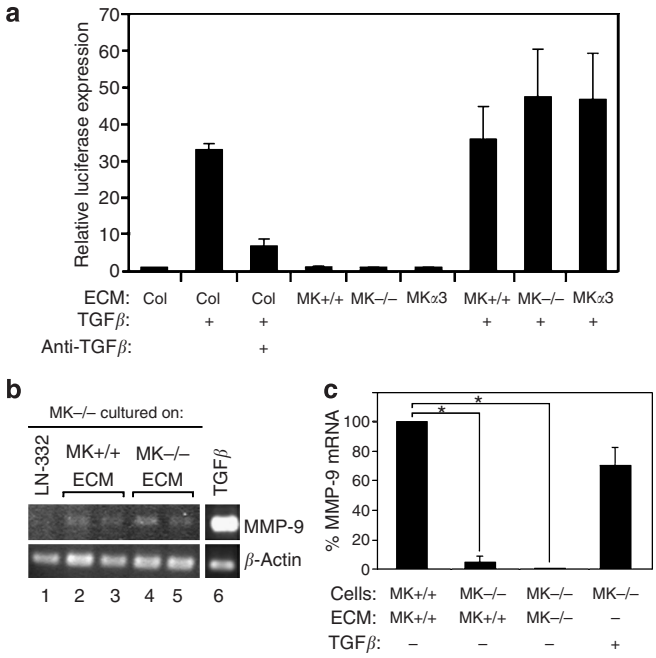

Figure 6. Expression of integrin α3β1 is not correlated with enhanced bioavailability of TGFβ in the ECM. (**a**) Collagen substrates were conditioned by MK cells and prepared as described in Materials and Methods. Control collagen-coated dishes (Col) or MK cell-conditioned ECMs were then incubated for an additional 24 hours in serum-free medium, plus or minus 10 ng/ml TGFβ, as indicated. Anti-TGFβ neutralizing antibody (30 μg/ml) was included where indicated. ECMs were rinsed extensively and assayed for levels of active TGFβ by measuring the induction of the PAI-1 promoter-luciferase reporter gene in MLEC cells, as described previously (Annes *et al.*, 2004). Data are the mean ± SEM; $n = 3$ or more experiments. (**b**) MK−/− cells were seeded onto LN-332 ECM that was conditioned by MK+/+ cells (duplicate lanes 2 and 3) or MK−/− cells (duplicate lanes 4 and 5), then cultured for 48 hours before RT-PCR analysis of MMP-9 and β-actin mRNA levels. Negative and positive controls, respectively, included MK−/− cells that were seeded onto unconditioned LN-332 ECM in the absence (lane 1) or presence of 1 ng/ml TGFβ (lane 6). (**c**) Real-time RT-PCR was performed to quantify MMP-9 mRNA expression in MK+/+ or MK−/− cells (cells) sub-cultured on LN-332 ECM that was conditioned as indicated (ECM). As a control, MK−/− cells were treated with 1 ng/ml TGFβ (+TGFβ). MMP-9 mRNA levels, after normalization to β-actin mRNA, are indicated as a percentage of the level in MK+/+ cells cultured on their own ECM in the absence of TGFβ. Data are mean ± SEM; $n = 3$; $^*P < 0.01$, one-way analysis of variance with Newman–Keuls *post hoc* test.

First, we confirmed that MLECs are responsive to active TGFβ under our experimental conditions. MLECs showed basal luciferase expression when seeded onto collagen (Figure 6a, Col). Luciferase expression was robustly induced in MLECs that were seeded onto collagen that had been pre-coated with active TGFβ (Figure 6a, Col, +TGFβ), and this induction was blocked by the addition of anti-TGFβ neutralizing antibody (Figure 6a, Col, +TGFβ, +anti-TGFβ). Next, we tested for the presence of active TGFβ in ECM substrates deposited by MK cells. When seeded onto collagen that had been conditioned for 2 days by MK+/+ cells, MK−/− cells, or MKα3 cells, MLECs showed a basal level of luciferase expression that was similar to that of untreated collagen (Figure 6a), indicating the absence of detectable levels of activated TGFβ. As a control, luciferase expression was induced by each of the three MK-conditioned ECMs if they were subsequently coated with active TGFβ (Figure 6a, MK ECMs +TGFβ), indicating that these ECMs did not contain inhibitors of TGFβ signaling. Consistently, culture of MK−/− cells on LN-332 ECM that had been conditioned by MK+/+ cells failed to induce MMP-9 mRNA expression, as assessed by either semi-quantitative RT-PCR (Figure 6b, lanes 2 and 3) or real-time RT-PCR (Figure 6c). As a control, MK+/+ cells continued to express high levels of MMP-9 mRNA when re-plated onto either their own ECM (Figure 6c) or ECM that had been conditioned by MK−/− cells (data not shown). Results in Figure 6 indicate that α3β1-dependent MMP-9 expression is not a result of α3β1-dependent differences in the bioavailability of ECM-associated TGFβ.

Integrin α3β1 potentiates induction of MMP-9 mRNA by active TGFβ in immortalized keratinocytes, but not in primary keratinocytes

Since α3β1 and endogenous TGFβ are both required for full induction of MMP-9 expression in MK cells (Iyer *et al.*, 2005) (Figure 1), but we found no evidence that α3β1 regulates the expression or bioavailability of endogenous TGFβ, our findings collectively suggest that α3β1 augments the ability of MK cells to respond to active TGFβ. Indeed, results in Figure 2 show that exogenously added active TGFβ can induce MMP-9 expression in the absence of α3β1 only when it is provided at relatively high concentrations. To directly test whether α3β1 further enhances induction of MMP-9 by exogenous TGFβ, MMP-9 expression was compared between α3β1-expressing and α3β1-deficient MK cells in the presence or absence of TGFβ. As expected, treatment with 10 ng/ml TGFβ enhanced the levels of MMP-9 protein and mRNA above baseline levels in both MK+/+ cells and MK−/− cells, as assessed by gelatin zymography and RT-PCR, respectively (Figure 7a and b). Importantly, however, TGFβ-mediated induction of MMP-9 protein and mRNA was greatly enhanced in α3β1-expressing MK cells (Figure 7a and b),

indicating that α3β1 potentiates the induction of MMP-9 in response to TGFβ. Quantification of the individual and combined effects of TGFβ and α3β1 on MMP-9 mRNA levels using real-time RT-PCR showed that MMP-9 mRNA was induced in MK−/− cells to similar levels, by either TGFβ treatment or stable expression of human α3 integrin (Figure 7c; compare bars for MK−/−, +TGFβ and MKα3, −TGFβ). However, TGFβ and α3β1 cooperated for maximal induction of MMP-9 mRNA (Figure 7c; see bars for MK+/+, +TGFβ and MKα3, +TGFβ).

Previously, we showed that α3β1-dependent MMP-9 expression is acquired by immortalized keratinocytes (DiPersio *et al.*, 2000). Therefore, we next tested whether α3β1 also enhances TGFβ-mediated induction of MMP-9 in freshly isolated, non-immortalized keratinocytes. MMP-9 mRNA was induced in primary keratinocytes by TGFβ concentrations ranging from 0.5 to 10 ng/ml (Figure 7d, and data not shown). However, in contrast with immortalized MK cells, the induction of MMP-9 mRNA by TGFβ in primary keratinocytes was similar in the presence (α3+/+) or absence (α3−/−) of α3β1 (Figure 7d), indicating that cooperative effects of TGFβ and α3β1 are associated with the immortalized phenotype.

We next tested whether α3β1-deficient MK−/− cells were generally deficient in TGFβ-mediated signal transduction. Western blot analysis showed that basal levels of TGFβ receptors I and II were similar in MK cells that express or lack α3β1 (Figure 8a). Furthermore, TGFβ receptor function was intact in both MK−/− and MKα3 cells under our experimental conditions, as indicated by similar robust Smad phosphorylation in response to TGFβ treatment (Figure 8b). Consistently, TGFβ enhanced expression of a PAI-1 promoter/*luciferase* reporter gene (Allen *et al.*, 2005) to a modest, but statistically significant and comparable extent in MK−/− cells and MKα3 cells, indicating that TGFβ signaling to the PAI-1 promoter, which involves Smads (Dennler *et al.*, 1998), was not α3β1-dependent (Figure 8c). Taken together, results in Figure 8 indicate that TGFβ-mediated Smad signaling is not directly involved in α3β1/TGFβ-mediated regulation of MMP-9 expression.

A recent study in breast cancer cells identified Smad-independent TGFβ signaling that occurs through a pathway that involves Src (Galliher and Schiemann, 2007), and we showed previously that α3β1 regulates Src kinase signaling in immortalized MK cells (Choma *et al.*, 2007). Treatment of MKα3 cells with the SFK inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) completely abrogated the inductive effect of TGFβ on MMP-9 mRNA expression, indicating a requirement for SFK signaling in this regulation (Figure 9a). Potent suppression of MMP-9 mRNA expression by PP2 was confirmed in TGFβ-treated MK+/+

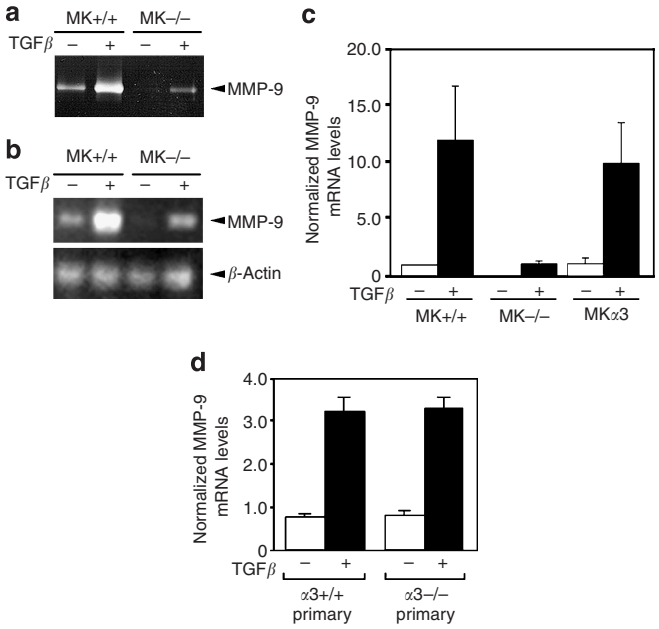

Figure 7. The ability of integrin α3β1 to enhance TGFβ-mediated MMP-9 induction is acquired by immortalized MK cells. (**a**–**c**) α3β1-deficient MK cells (MK−/−) or α3β1-expressing MK cells (MK+/+ or MKα3) were grown on LN-332 ECM in serum-free medium in the presence (+lanes) or absence (−lanes) of 10 ng/ml TGFβ. (**a**) Gelatin zymography was performed after 48 hours to assess MMP-9 secretion. Both RT-PCR (**b**) and real-time RT-PCR (**c**) were performed after 24 hours to assess MMP-9 mRNA, or β-actin mRNA as a control. Results in (**a**) and (**b**) are representative of at least three experiments. Graph in (**c**) shows relative MMP-9 mRNA levels, after normalization to β-actin mRNA, compared with MK+/+ cells in the absence of TGFβ (set at 1.0); data are mean±SEM; $n=3$. (**d**) Non-immortalized, primary keratinocytes were isolated from epidermis of wild-type (α3+/+) or α3-null (α3−/−) neonatal mice and cultured in the presence or absence of TGFβ (10 ng/ml) for 24 hours, and MMP-9 mRNA was quantified by real-time RT-PCR. Graph shows relative MMP-9 mRNA levels (arbitrary units) after normalization to β-actin mRNA; data are mean±SEM; α3+/+, $n=10$; α3−/−, $n=4$. Inductive effects of TGFβ were significant ($P<0.001$, two-way analysis of variance), but there were no significant differences in this inductive effect between α3+/+ and α3−/−.

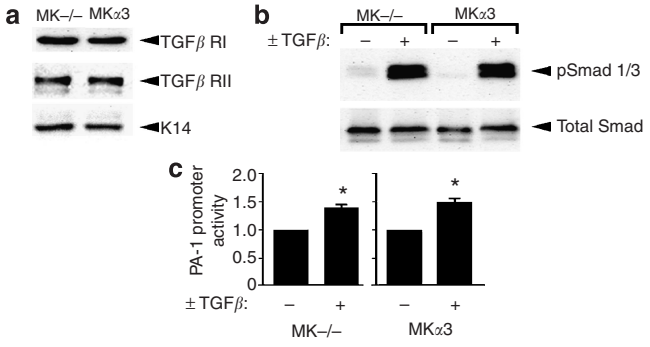

Figure 8. TGFβ-mediated Smad signaling and PAI-1 promoter induction is intact in α3β1-deficient MK−/− cells. (**a**) Western blots show similar expression of TGFβ receptors I (TGFβ RI) and II (TGFβ RII) in MK−/− cells and MKα3 cells; keratin 14 (K14) is shown as a loading control. (**b**) MK−/− cells or MKα3 cells on LN-332 ECM were serum-starved for 24 hours, then treated for 1 hour with TGFβ (10 ng/ml) or left untreated, as indicated. Equal protein was immunoblotted for phosphorylated Smad1/3, or total Smad protein as a loading control. Results in (**a**) and (**b**) are representative of at least three separate experiments. (**c**) MK−/− cells or MKα3 cells grown on LN-332 ECM were transfected with a PAI-1 promoter/luciferase reporter plasmid, then cultured for 24 hours in the presence of absence of TGFβ (10 ng/ml), and cell lysates were assayed for luciferase expression (arbitrary units). Data are the mean±SEM, $n=6$ in three separate experiments; *$P<0.01$, *t*-test.

cells by quantitative real-time RT-PCR (Figure 9b). SFK inhibition also reduced basal MMP-9 mRNA levels in α3β1-expressing MK cells that were not treated with exogenous TGFβ (data not shown), suggesting that a Src kinase is also required for maintenance of basal MMP-9 expression by endogenous TGFβ (Figure 1).

TGFβ and α3β1 cooperatively enhance the induction of MMP-9 by oncogenic Ras

Cooperative signaling through TGFβ and oncogenic Ras is required for the maintenance of EMT in many epithelial cell types (Oft *et al.*, 1996; Zavadil and Bottinger, 2005). Oncogenic activation of Ras leads to the induction of MMP-9 through transcriptional activation of the MMP-9 promoter (Gum *et al.*, 1996; Sehgal and Thompson, 1999). We showed previously that α3β1 potentiates the induction of MMP-9 by RasV12 in immortalized keratinocytes by promoting post-transcriptional mRNA stability, which was necessary for accumulation of MMP-9 mRNA following RasV12-mediated transcriptional stimulation (Iyer *et al.*, 2005). Given that cooperative induction of MMP-9 by α3β1 and TGFβ was acquired by immortalized keratinocytes, we next wanted to test if TGFβ and α3β1 can cooperate to enhance the induction of MMP-9 by oncogenic Ras. To this end, we compared the individual and combined effects of α3β1 and TGFβ on RasV12-mediated MMP-9 induction. First, MK+/+ cells cultured on LN-332 ECM were infected with RasV12 adenovirus, or left uninfected as a control, then either treated with TGFβ or left untreated. Gelatin zymography showed that RasV12 and TGFβ each independently induced MMP-9 protein levels in MK+/+ cells, and in combination they had a greater effect (Figure 10a, lanes 1–4). Similar effects were seen on MMP-9 mRNA levels (Figure 10b, lanes 1–4).

Assessment of MK−/− cells confirmed our previous findings that efficient induction of MMP-9 by RasV12 was dependent on α3β1 (Iyer *et al.*, 2005), since RasV12 failed to induce significant levels of MMP-9 protein secretion (Figure 10a, lane 6) and had a minimal effect on MMP-9 mRNA levels (Figure 10b, lane 6). As expected, TGFβ alone modestly induced MMP-9 protein and mRNA (Figure 10a and b, lane 7). Infection of MK−/− cells with RasV12, in combination with TGFβ treatment, further enhanced MMP-9 expression (Figure 10a and b, lane 8), demonstrating that TGFβ, like α3β1 integrin, potentiates RasV12-mediated induction of MMP-9. However, the highest level of MMP-9 induction by RasV12 was observed when both α3β1 and TGFβ were present (Figure 10a and b, lane 4), indicating cooperative effects between the integrin and growth factor.

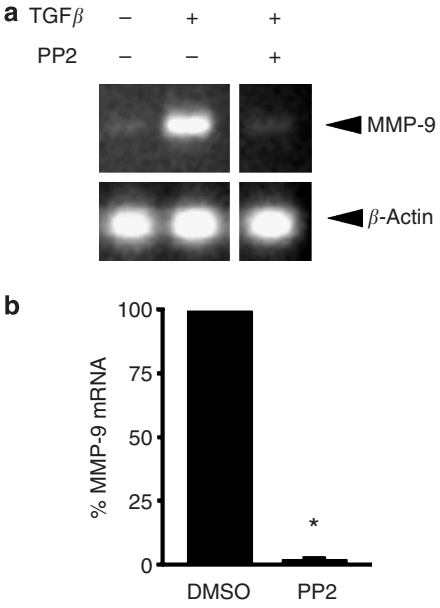

Figure 9. SFK activity is required for TGFβ-mediated induction of MMP-9. (**a**) α3β1-expressing MKα3 cells on LN-332 ECM were pretreated with serum-free medium containing the SFK inhibitor PP2 (10 μm) or dimethylsulfoxide (DMSO) only as a control, then cultured for an additional 24 hours in the presence or absence of 10 ng/ml TGFβ, as indicated. RT-PCR was performed after 24 hours to assess MMP-9 mRNA, or β-actin mRNA as a control. Note that, for this experiment, the PCR cycle number was adjusted so that basal MMP-9 mRNA levels are just detectable in untreated cells, so that TGFβ-mediated induction is readily evident. (**b**) α3β1-expressing MK+/+ cells were treated with 10 ng/ml TGFβ and grown as in (**a**) in the presence of PP2, or DMSO as a control, and MMP-9 mRNA was quantified by real-time RT-PCR. Graph shows MMP-9 mRNA levels in PP2-treated cells as a percentage of levels in control cells, after normalization to β-actin mRNA. Data are the mean ± SEM, $n = 3$; $^*P < 0.001$, *t*-test.

DISCUSSION

Studies in cultured keratinocytes have shown that integrin α3β1 regulates several functions associated with EMT and/or

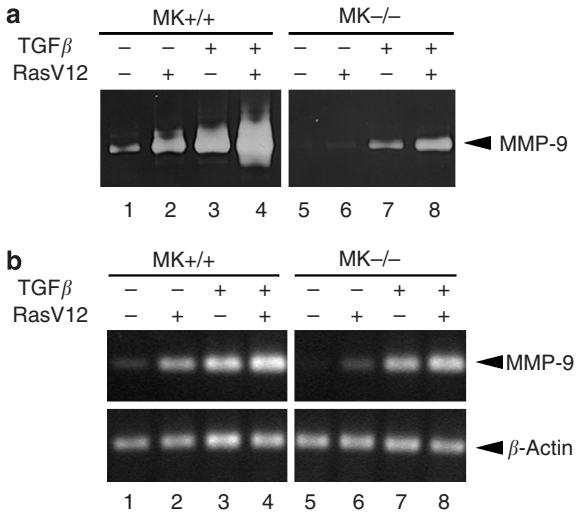

Figure 10. TGFβ and α3β1 cooperatively enhance MMP-9 induction in response to oncogenic RasV12. (**a** and **b**) MK+/+ cells (lanes 1–4) or MK−/− cells (lanes 5–8) were grown on LN-332 ECM and either left uninfected (lanes 1, 3, 5, and 7) or infected with RasV12 adenovirus (lanes 2, 4, 6, and 8). Cells were then cultured in serum-free medium for an additional 48 hours in the presence (lanes 3, 4, 7, and 8) or absence (lanes 1, 2, 5, and 6) of TGFβ (10 ng/ml). (**a**) Cell culture medium was collected and assayed by gelatin zymography for MMP-9 protein levels, and (**b**) total RNA was isolated and assayed by RT-PCR for MMP-9 and β-actin mRNA levels. Results are representative of three separate experiments.

wound healing, including ECM deposition and organization (deHart *et al.*, 2003; Hamelers *et al.*, 2005), cell polarization and migration (Choma *et al.*, 2004, 2007; Frank and Carter, 2004), and cell survival (Manohar *et al.*, 2004). Cooperative interactions between TGFβ and α3β1, or other integrins, have been implicated in the regulation of motility or invasiveness of keratinocytes and carcinoma cells (Gailit *et al.*, 1994; Zambruno *et al.*, 1995; Giannelli *et al.*, 2002; Decline *et al.*, 2003; Galliher and Schiemann, 2007). In this study, we provide early evidence of a role for α3β1 in enhancing the TGFβ-mediated induction of MMP-9 gene expression in immortalized keratinocytes. Our results show that reduced MMP-9 expression in α3β1-deficient MK−/− cells is not due to a reduction in basal expression of either TGFβ (Figure 4) or of TGFβ receptors I and II (Figure 8a). At the same time, endogenous TGFβ was required for full induction of MMP-9 in α3β1-expressing MK+/+ cells (Figure 1b), but it was not sufficient to maintain MMP-9 expression in MK−/− cells. Indeed, relatively high concentrations of exogenous TGFβ were required to induce MMP-9 to a detectable level in the latter cells (Figure 2), and the presence of α3β1 further enhanced this inductive response (Figure 7a–c). Taken together, these findings indicate that α3-null MK−/− cells are deficient in a signaling pathway that promotes TGFβ-mediated MMP-9 expression.

It is well established that TGFβ is deposited into the ECM as part of a latent complex that needs to be activated to initiate a signaling response (Sheppard, 2005). We did not detect differences in bioavailable TGFβ between ECM from α3β1-expressing MK cells or α3-null MK−/− cells, and α3-null MK−/− cells were unable to produce MMP-9 in response to ECM that was conditioned by α3-positive MK+/+ cells (Figure 6). It is unlikely that these results simply reflect insufficient levels of TGFβ in the ECM, since blocking endogenous TGFβ reduced MMP-9 expression in MK+/+ cells (Figure 1), while endogenous TGFβ secreted into the culture medium was not sufficiently high in these cells to induce MMP-9 (Figure 5). Rather, these results suggest that MK−/− cells are unable to increase MMP-9 production in response to ECM-associated TGFβ. Binding of integrin αvβ6 to an arginine–glycine–aspartic motif in latency-associated protein activates latent, ECM-associated TGFβ (Munger *et al.*, 1999), and αv integrins are essential for TGFβ-mediated signaling *in vivo* (Yang *et al.*, 2007). However, it is very unlikely that α3β1 functions similarly, since it binds poorly, or not at all, to arginine–glycine–aspartic ligands (Hynes *et al.*, 1989; Weitzman *et al.*, 1993; Delwel *et al.*, 1994), and a similar mechanism for β1 integrins was not supported (Munger *et al.*, 1999). It is also unlikely that αvβ6 has a direct role in α3β1/TGFβ-mediated induction of MMP-9, since TGFβ-mediated Smad signaling was intact in MKα3−/− cells under our experimental conditions (Figure 8), indicating that αvβ6-dependent TGFβ activation is neither compromised in these cells nor sufficient for induction of MMP-9. In addition, our finding that α3β1 enhances MMP-9 induction by exogenous, pre-activated TGFβ (Figure 7) suggests that α3β1 contributes to a TGFβ signaling pathway at a point downstream of TGFβ activation.

TGFβ can signal through Smad-dependent or Smad-independent pathways (Derynck and Zhang, 2003; Moustakas and Heldin, 2005). Our findings in MK cells suggest that TGFβ induces MMP-9 expression through a non-Smad, SFK-dependent mechanism (Figures 8 and 9). Together with our previous report that α3β1 activates Src signaling pathways in MK cells (Choma *et al.*, 2007), these results suggest that a Src family member may link α3β1 to TGFβ-mediated induction of MMP-9 mRNA. These findings are intriguing in light of a recent study in breast cancer cells, which showed that Src-mediated phosphorylation of the TGFβ type II receptor was required for TGFβ to activate mitogen-activated protein kinase pathways (i.e. p38), but not for activation of Smad pathways (Galliher and Schiemann, 2007). Future studies will explore whether similar mechanisms of Src-mediated TGFβ signaling exist in MK cells.

TGFβ regulates many genes at the level of post-transcriptional mRNA stability (Dibrov *et al.*, 2006), including MMP-9 in prostate cancer cells (Sehgal and Thompson, 1999). Our findings that integrin α3β1 also induces MMP-9 mRNA stability in immortalized keratinocytes (Iyer *et al.*, 2005), and that neither α3β1 nor TGFβ induced the MMP-9 promoter in these cells (Iyer *et al.*, 2005) (Figure 3), suggest that TGFβ and α3β1 may cooperatively regulate a signaling pathway that promotes MMP-9 mRNA stability. Cooperative signaling could occur via interactions of a TGFβ receptor complex with α3β1, as described for other growth factor receptors and integrins (ffrench-Constant and Colognato, 2004; Guo *et al.*, 2006). Alternatively, distinct signaling events that are activated independently by TGFβ or α3β1 may converge on a common intracellular pathway. Extracellular signal-regulated kinase (ERK) is a potential downstream effector of TGFβ/α3β1-mediated signaling that induces MMP-9 expression, since TGFβ activates ERK in cell culture models of EMT (Zavadil and Bottinger, 2005), and ERK is both activated by α3β1 and required for MMP-9 expression in MK cells (Manohar *et al.*, 2004; Iyer *et al.*, 2005). Consistently, a recent study showed that autocrine TGFβ signaling through the type I receptor ALK5 leads to mitogen-activated protein kinase/ERK-dependent induction of MMP-9 mRNA in breast cancer cells (Safina *et al.*, 2007). Furthermore, we previously showed that SFK signaling is required for α3β1-mediated ERK activation in MK cells (Choma *et al.*, 2007), suggesting that a SFK may link α3β1 and TGFβ to an ERK signaling pathway that promotes MMP-9 expression. p38 is another potential effector for this regulation, since it is involved in TGFβ-mediated EMT (Bakin *et al.*, 2002; Zavadil and Bottinger, 2005), and cooperative interactions between β1 integrins and TGFβ can activate p38 in mouse mammary epithelial cells (Bhowmick *et al.*, 2001).

α3β1-mediated induction of MMP-9 gene expression is a pathway that is acquired during keratinocyte immortalization, indicating changes in α3β1 function during EMT (DiPersio *et al.*, 2000). It is well known that TGFβ switches from a tumor suppressor to a tumor promoter during EMT and skin carcinogenesis (Derynck *et al.*, 2001; He *et al.*, 2001; Wang, 2001; Wakefield and Roberts, 2002), and TGFβ-mediated induction of MMP-9 was similarly acquired in

prostate cancer cells (Sehgal and Thompson, 1999). In this study, we showed that cooperative induction of MMP-9 by α3β1 and TGFβ was acquired by keratinocytes that were immortalized through expression of SV40 large T antigen. This regulation was not specific to immortalization by large T antigen, since we have observed similar regulation in keratinocytes that were immortalized through p53 null-mutation (J Lamar and CM DiPersio, unpublished). Therefore, the acquisition of this regulation occurs as part of the immortalized phenotype, suggesting a signaling switch that occurs during EMT. It is well established that TGFβ and oncogenic Ras cooperate to maintain EMT in epithelial cells (Zavadil and Bottinger, 2005). Our results suggest that TGFβ and α3β1 potentiate maximal MMP-9 induction in response to RasV12, possibly reflecting an important role for cooperation between TGFβ, integrin α3β1, and oncogenic Ras in the maintenance of MMP-9 expression during epidermal tumor growth and/or invasion.

MATERIALS AND METHODS

Culture of primary keratinocytes and MK cells

Primary keratinocytes were isolated from epidermis of wild-type (α3+/+) or α3-null (α3−/−) neonatal mice and cultured as described previously (DiPersio *et al.*, 1997). MKs were immortalized by SV40 large T antigen expression as described previously (DiPersio *et al.*, 2000). The MK+/+ cell line (MK-1.16) and MK−/− cell line (MK-5.4.6) were derived from keratinocytes isolated from wild-type or α3-null mice, respectively (DiPersio *et al.*, 2000). MK−/− cells stably transfected with human α3 (MKα3 cells) express high levels of α3β1 on the cell surface (Iyer *et al.*, 2005). MK growth medium consisted of Eagle's minimum essential medium (BioWhittaker, Walkersville, MD) supplemented with 4% fetal bovine serum (BioWhittaker) from which Ca^{2+} had been chelated, 0.05 mM $CaCl_2$, 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 2×10^{-9} M T3, 10 units/ml interferon-γ (INFγ; (Sigma, St Louis, MO), 10 ng/ml epidermal growth factor, 100 units/ml penicillin, 100 μg/ml streptomycin, and L-glutamine (Invitrogen Corporation, Carlsbad, CA). MK cell lines were maintained at 33°C, 8% CO_2, on tissue culture plates coated with 30 μg/ml denatured rat tail collagen (BD Biosciences, Bedford, MA). For experiments, MK cells were sub-cultured on LN-332 ECM prepared from the SCC-25 squamous cell carcinoma line, as described previously (DiPersio *et al.*, 2000). We showed previously that α3-null MK−/− cells adhere efficiently to LN-332 ECM through integrin α6β4 (DiPersio *et al.*, 1997, 2000). All experiments and reagents described in this study were approved by the Institutional Biosafety Committee of Albany Medical College. All animal studies were approved by the Institutional Animal Care and Use Committee of Albany Medical College.

Antibodies and western blotting

MK cells were cultured on LN-332 ECM for 24–48 hours, in the presence or absence of TGFβ as indicated in the figure legends. Cells were then lysed in cell lysis buffer (Cell Signaling Technology, Beverly, MA), and protein concentrations were quantified using the BCA Protein Assay kit (Pierce, Rockford, IL). Equal protein was assayed by immunoblot using antibodies against the following proteins: TGFβ receptor I (R&D Systems, Minneapolis, MN), TGFβ receptor II (Santa Cruz Biotechnology, Santa Cruz, CA), keratin 14 (Covance, Richmond, CA), phosphorylated Smad1 (Ser463/465)/3(Ser423/425) (Cell Signaling Technology), and total Smad2/3 (Cell Signaling Technology).

Assessment of MMP-9 regulation by TGFβ

To test if exogenous TGFβ promotes MMP-9 expression, active TGFβ (R&D Systems) was added to serum-free cell culture medium for 24 hours at the concentrations indicated in the figure legends. In some experiments, cells were pretreated for 5 hours with the SFK inhibitor PP2 (10 μm; Calbiochem, San Diego, CA) or with dimethylsulfoxide only as a control, as described previously (Choma et al., 2007), then cultured for an additional 24 hours with TGFβ in the presence or absence of PP2. To test if blocking endogenous TGFβ inhibits MMP-9 expression, cells were cultured for 24 hours in serum-free medium containing the indicated concentration of pan-specific TGFβ-neutralizing antibody (R&D Systems). In some experiments, MK cells were also infected with RasV12 adenovirus, as described previously (Iyer *et al.*, 2005). MMP-9 mRNA or protein expression was determined by RT-PCR or gelatin zymography, respectively, as described below.

Assessment of MMP-9 induction by MK-conditioned culture medium or ECM

To generate MK cell-conditioned media, MK+/+ or MK−/− cells were cultured at high density on LN-332 ECM in serum-free medium, and after 24 hours the conditioned medium was collected and centrifuged to remove debris. MK cell-conditioned medium, or control serum-free medium, plus or minus 10 ng/ml TGFβ, was added to MK−/− cells on LN-332 ECM for 24 hours. Total RNA was collected and assayed by RT-PCR for MMP-9 and β-actin mRNA, as described below.

To generate MK cell-conditioned collagen substrate, MK+/+ or MK−/− cells were cultured in full-growth medium on 12-well plates pre-coated with 30 μg/ml denatured rat-tail collagen. After 48 hours, the MK cells were removed using 5 mM ethylenediaminetetraacetic acid, and the remaining ECM was rinsed extensively and incubated an additional 24 hours in serum-free medium, plus or minus 10 ng/ml TGFβ. As a control, unconditioned collagen substrate was similarly incubated in serum-free medium, plus or minus 10 ng/ml TGFβ. As a readout to detect active TGFβ in MK cell-conditioned or control ECMs, transformed MLECs stably transfected with a reporter plasmid containing the luciferase gene downstream of a TGFβ-responsive PAI-1 promoter were obtained from Dr Daniel Rifkin and used as described previously (Abe *et al.*, 1994). Briefly, 5×10^5 cells MLECs were cultured on either control or MK cell-conditioned collagen in DMEM, 10% fetal bovine serum for 24 hours. Where indicated as a control, function-blocking anti-TGFβ antibody was added to wells 30 minutes before seeding of MLECs. Cell lysates were assayed for relative luciferase activity using the Luciferase Assay System (Promega, Madison, WI), as described below.

To generate MK cell-conditioned LN-332 ECM, MK+/+ or MK−/− cells were cultured in full-growth medium on LN-332 ECM, and after 48 hours the MK cells were removed using 5 mM ethylenediaminetetraacetic acid and the remaining ECM was rinsed extensively. Fresh MK+/+ or MK−/− cells were seeded onto MK cell-conditioned LN-332 ECM in full-growth medium and allowed to recover overnight. MK cells were then rinsed extensively and incubated in serum-free growth medium, plus or minus 10 ng/ml

TGFβ, for an additional 24 hours. Total RNA was collected and assayed by RT-PCR for MMP-9 and β-actin mRNA, as described below.

Gelatin zymography

MK cells were cultured in serum-free medium for 48 hours, and culture media were collected for gelatin zymography. MMPs were concentrated from culture media by binding overnight at 4°C to gelatin–agarose beads (Sigma), recovery by centrifugation, and elution in zymography sample buffer (final: 2.25% SDS, 9% glycerol, 45 mM Tris pH 6.8, bromophenol blue), then resolved by non-reducing SDS/PAGE on 10% polyacrylamide gels impregnated with 1 mg/ml gelatin (Sigma), as described previously (DiPersio *et al.*, 2000).

RT-PCR

Total cellular RNA was isolated from MK cells using the Purescript RNA Isolation kit (Gentra Systems, Minneapolis, MN) or Trizol Reagent (Invitrogen Corporation), then reverse transcribed to produce cDNA template using the First-Strand cDNA Synthesis kit (Promega). PCR reactions were carried out in 12.5 μl PCR REDTaq ReadyMix (Sigma) with 5 μl of cDNA and 0.4 μM of each primer. PCR primers and conditions for amplification of MMP-9 and β-actin were described previously (Iyer *et al.*, 2005). For some experiments, amplification of β-actin was performed using the real-time RT-PCR primers described below. PCR primers for TGFβ were as described (Romieu *et al.*, 1997), and generate a 200-bp product: forward primer, 5′-ACCGCAACAACGCCATCTAT-3′; reverse primer, 5′-GTAACGCCAGGAATTGTTGC-3′. PCR conditions for amplification of TGFβ were 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, 26 amplification cycles. PCR products were resolved on agarose gels, stained with ethidium bromide, and visualized using a Bio-Rad Gel-Doc 2000. Signals were quantified using Quantity One software (Bio-Rad, Hercules, CA). For quantitative real-time RT-PCR analysis of MMP-9 and β-actin mRNA expression, PCR reactions were carried out in 10 μl IQ SYBR Green Supermix (Bio-Rad) with 5 μl of cDNA and 1 μM of each primer. Analysis was performed using MyiQ real-time PCR detection system according to the manufacturer's instructions (Bio-Rad). PCR conditions were 94°C for 3 minutes, followed by 40 cycles of 94°C for 30 seconds, 54°C for 30 seconds, 72°C for 30 seconds. PCR primers for MMP-9 generate a 67-bp product: forward primer, 5′-CAGCTGGCAGAGGCATACTTG-3′; reverse primer, 5′-GCTTCTCTCCCATCATCTGGG-3′. PCR primers for β-actin generate a 203-bp product: forward primer, 5′-TGGTGGTACCACCATGTACC-3′; reverse primer, 5′-AGGGGCCGGACTCATCGTACT-3′. Melting curves were generated for each reaction product.

Analysis of transfected MMP-9 and PAI-1 promoters

For MMP-9 promoter assays, an MMP-9 promoter/firefly luciferase reporter plasmid, was a generous gift from Dr Y. Sasaguri (University of Occupational and Environmental Health, Kitakyushu, Japan) and contained a 1,868-bp DNA fragment of the MMP-9 promoter region (−1,879 to −12 from the transcription start site) cloned upstream of the *luciferase* gene in the pGL3 vector (Promega), as described previously (Shimajiri *et al.*, 1999). MK cells seeded on LN-332 ECM were co-transfected with the MMP-9 promoter/luciferase reporter plasmid and a TK promoter/*Renilla luciferase* control plasmid (Promega) at a 50:1 ratio using lipofectamine, then cultured for 48 hours in serum-free medium, plus or minus 10 ng/ml TGFβ. As a positive control for MMP-9 promoter induction, MK cells were infected with RasV12 adenovirus before transfection, as described previously (Iyer *et al.*, 2005). For PAI-1 promoter assays, MK cells were co-transfected with the *Renilla* control plasmid and a PAI-1 promoter/firefly *luciferase* reporter plasmid, a gift from Dr P. Higgins (Albany Medical College, Albany, NY), which contained the PAI-1 promoter (nucleotides −806 to +72) cloned upstream of the *luciferase* gene, as described previously (Allen *et al.*, 2005). Transfected cells were then cultured for 24 hours in the presence or absence of 10 ng/ml TGFβ, as described above. Cell lysates were assayed for luciferase expression using the Dual-Luciferase Reporter Assay kit (Promega). Luciferase expression was measured in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA), and expression from *luciferase* reporter plasmids was normalized to that from the control promoter/*Renilla luciferase* control plasmid for each sample.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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